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ARTICLE

^{1}H, ^{13}C and ^{15}N NMR assignments of Duck HBV apical stem loop of the epsilon encapsidation signal

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Abstract The replication of Hepatitis B virus is initiated by binding of its reverse transcriptase to the apical stem loop and primer loop of epsilon. Here, we present the $^{1}H/^{13}C/^{15}N$ NMR assignments of the bases and sugars of the 29 residues apical stem loop of Duck HBV epsilon.

Keywords HBV · RNA · Duck · Apical loop

Biological context

The Hepatitis B virus is the most common cause of liver infection in the world, with 300 million people worldwide estimated to be chronically infected. No efficient elimination of HBV in effected patients exists as yet. HBV is a member of the *Hepadnaviridae* family consisting of the hepatotropic DNA viruses, which also includes related animal viruses such as duck HBV and heron HBV. The genome of HBV is a small (3.2 kb), relaxed circular, partially double stranded DNA genome which replicates by reverse transcription of an RNA intermediate, the pregenomic RNA (Beck and Nassal 2007; Girard et al. 2007). The pregenomic RNA is transported into the cytoplasm and encapsidated into immature core particles together with HBV reverse transcriptase (RT). Binding of the viral RT to the encapsidation signal, epsilon, ε, a conserved 60nt bulged RNA located at the 5′ end of the RNA pregenome, triggers encapsidation. Subsequently, a 4nt DNA primer is synthesized using the ε-primer loop as template. The resulting complex translocates to a 3′-proximal ε RNA element of the pgRNA, where full-length DNA synthesis is started using the 4-nt DNA as primer (Flodell et al. 2006; Girard et al. 2007; Petzold et al. 2007). Although the structural basis and sequence requirements for RT-ε binding and priming are emerging, several questions remain and a full understanding of the molecular basis for the specific interactions between P and ε awaits high-resolution structural and thermodynamic data. The importance of high-resolution structural data is underlined by the NMR studies of the *human* HBV ε apical stem-loop, which showed that its conserved apical loop folds into a pseudo-triloop, whereas secondary structure programs predicted a hexaloop (Flodell et al. 2002, 2006). A functional in-vitro RT-ε replication system exists for Duck, but not for human HBV. The Duck RT-ε interaction is therefore best understood, although many conclusions can be extrapolated to Human HBV thanks to the close similarity of the two HBVs (Beck and Nassal 2007). We therefore study the human and Duck ε-RNAs in parallel. Here we present the NMR assignment of the Duck HBV ε apical stem-loop.

Methods and experiments

The RNA sequence was prepared as previously described (Girard et al. 2007). NMR samples were prepared in buffer (10 mM Na-Phosphate, pH 6.7, 0.1 mM EDTA) giving a 1.2 mM non-labelled sample, a 1.0 mM $^{13}C/^{15}N/^{2}H_{1',3',4',5',5''}$-U-labelled sample and a 0.7 mM fully $^{13}C/^{15}N$ labelled sample (Girard et al. 2007). Standard methods, such as 2D NOESY (in H_2O at 5°C and D_2O at 25°C), DQF-COSY (D_2O at 25°C), $^{1}H/^{15}N$ HMQC (H_2O at 5°C) and $^{1}H/^{13}C$ HSQC (D_2O at 25°C) spectra were used for initial

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assignments (Flinders and Dieckmann 2006; Girard et al. 2007; Wijmenga and van Buuren 1998). To get a more complete assignment a 3D selective ^{13}C NOESY was recorded as well as a straight-through H1′C1′(N)CbHb (b = base) correlation experiment recorded in a 4D fashion (H1′, C1′, Hb, and Cb evolution) to reduce overlap, a 3D out-and-back HCN correlation (sugar) and a straight-through 3D H1′(C1′)N(Cb)Hb correlation experimented complemented with region selective (H8/C8; H6/C6; H5/C5; H1′/C1′) 2D $^{1}H/^{13}C$ and $^{1}H/^{15}N$ HSQCs (Flinders and Dieckmann 2006; Girard et al. 2007; Wijmenga and van Buuren 1998). In addition, near complete sugar resonance assignment (H1′–H4′, C1′–C4′) was achieved via 2D and 3D experiments based on HCCH-TOCSY and a HCCH-COSY spectrum (Ampt, Tessari and Wijmenga, personal communication; Flinders and Dieckmann 2006; Wijmenga and van Buuren 1998). All experiments were carried out at 15°C on a 600 MHz Varian Inova spectrometer on the fully $^{13}C/^{15}N$ labelled sample in H_2O.

Spectra were processed using NMRpipe (Delaglio et al. 1995) and resonance assignment was performed with Sparky software (Kneller and Kuntz 1993).

Assignments and data deposition

The assignments have been deposited in the BMRB database under number 15656 with assignment tables at three different temperatures, 5°C, 15°C and 25°C. Initial assignment was carried out according to standard methods (Flinders and Dieckmann 2006; Wijmenga and van Buuren 1998). Spectra were recorded at 5°C in H_2O to establish the number of base pairs by assigning the imino's. At 25°C (in D_2O) the aromatic (H2, H5, H6, H8) and sugar (H1′, H2′) chemical shifts were determined by following the sequential walks in NOESY spectra. The sequential walks are interrupted at residue U25 due to dynamic movement of this residue. Sugar puckering modes were derived from observable/non observable H1′–H2′ coherences in the COSY spectrum (Girard et al. 2007). Spectra recorded at 15°C were used to verify the initial assignments as well as supplement the ^{13}C and ^{15}N chemical shifts and sugar-base correlation via 2D HSQC, 3D HCN, 3D HCNCH (Fig. 1) and 4D HC(N)CH spectra (Flinders and Dieckmann 2006; Wijmenga and van Buuren 1998). 2D $^{1}H/^{13}C$ selective region HSQC spectra were recorded in an IPAP fashion to

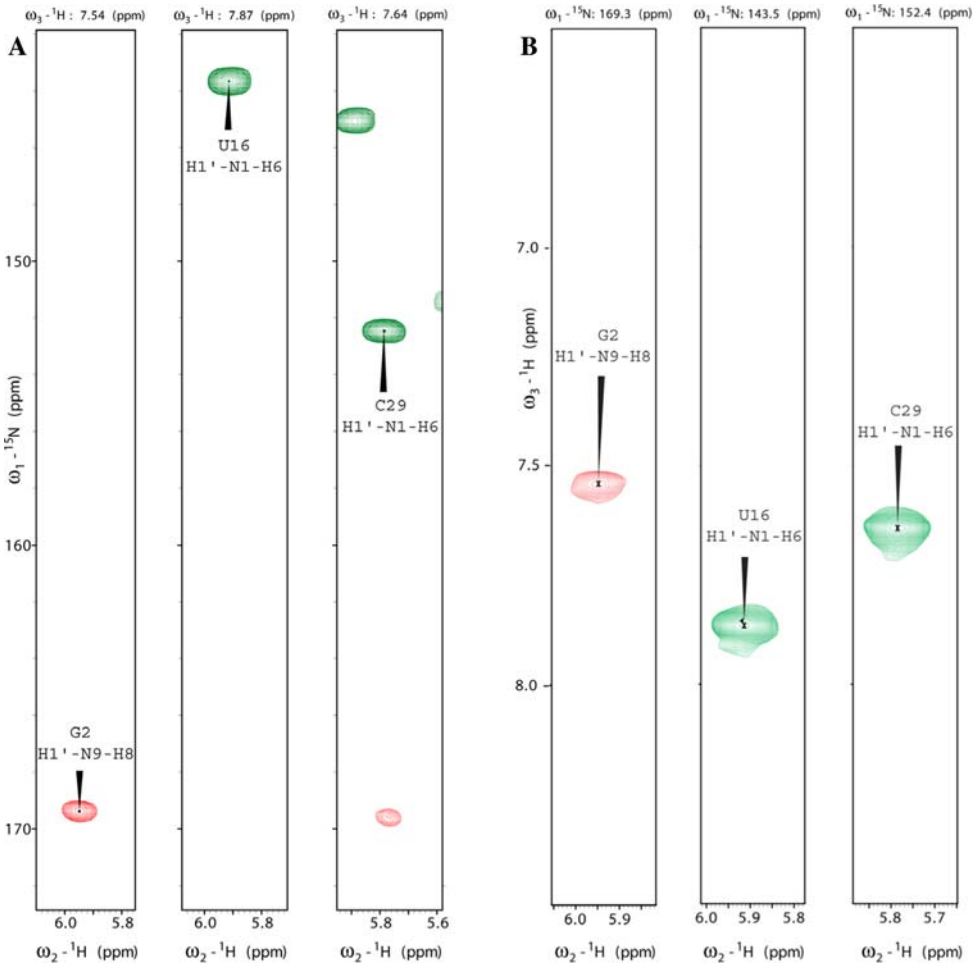

Fig. 1 Selected parts of the 3D HCNCH spectrum recorded at 15°C at 600 MHz on 0.72 mM fully $^{13}C/^{15}N$ labeled (10 mM Na-Phosphate Ph 6.7, 0.1 mM EDTA, 93:7 H_2O: D_2O) apical stem loop of Duck HBV. The spectrum provides N9/1-separated intra-residue H6/H8-H1′ correlations. (**a**) N1/9(ω_1)-H1′(ω_2) slices demonstrating how the N9(G/A) and/or N1 (U/C) shifts separate the H6/8-H1′ correlations of G/A, U and C residues, respectively. (**b**) H6/8(ω_1)-H1′(ω_1) slices showing N9/1-separated intra-residue H6/8-H1′ correlations; these slices can be directly compared with the H6/8-H1′ region of a NOESY spectrum and provide the unambiguous identification of the intra-residue correlations in the H6/8-H1′ region of the NOESY spectrum

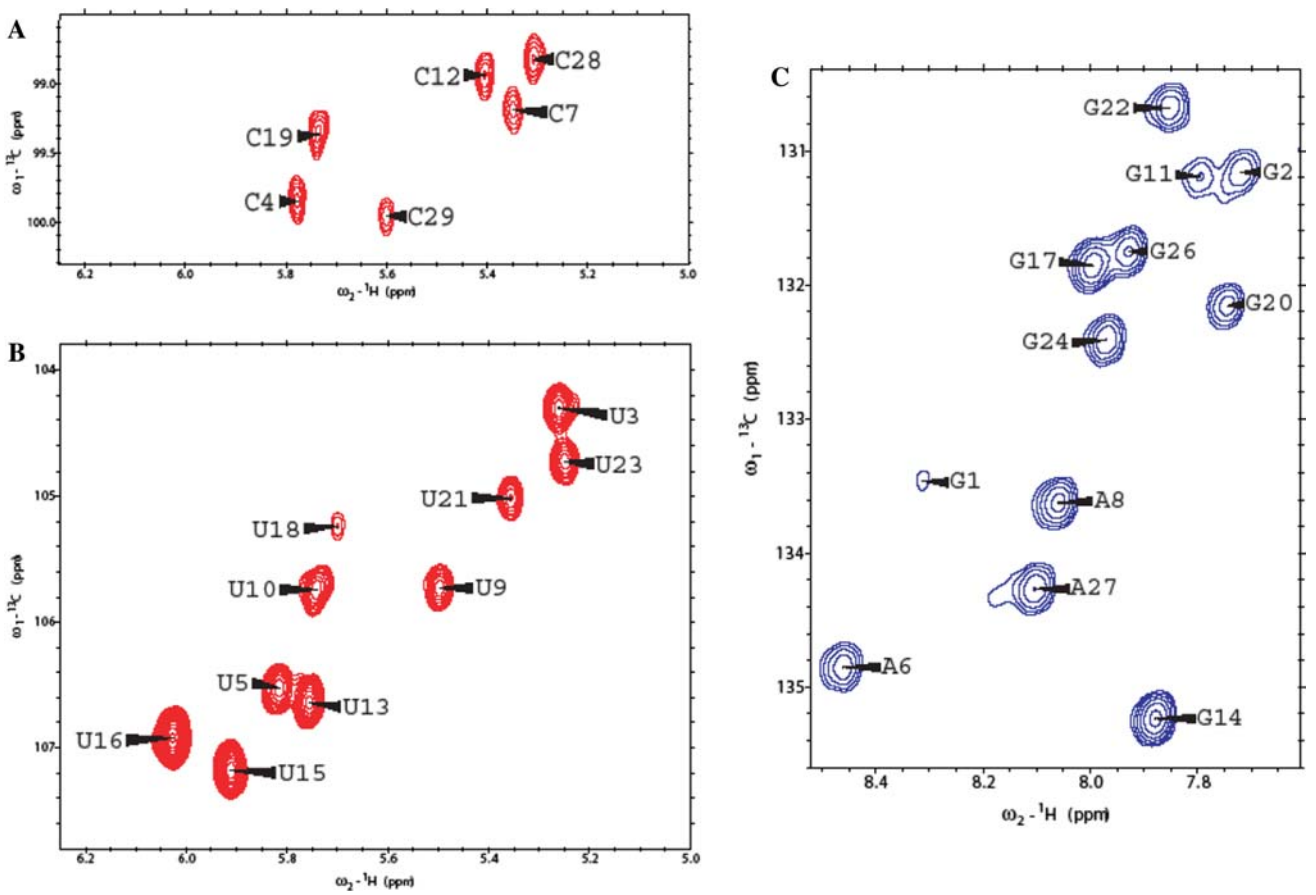

Fig. 2 Sum spectrum of the 2D IPAP $^{1}H/^{13}C$ CT HSQC spectrum recorded at 15°C at 600 MHz on 0.72 mM fully $^{13}C/^{15}N$ labelled (10 mM Na-Phosphate Ph 6.7, 0.1 mM EDTA, 93:7 H_2O: D_2O) apical stem loop of Duck HBV. Resonance assignments are indicated by sequence numbers and type of residue. (**a**) C5H5 Cytosine region, ^{1}H resonances should be shifted by ca. 0.15 ppm upfield to obtain the actual chemical shift or for exact calculation, the upfield shift can be derived from the J_{C5H5} couplings in Hz for each residue; the values are (in Hz), C4: 174.1, C7: 170.7, C12: 173.9, C19: 174.2, C28: 172.7, and C29: 173.6. (**b**) C5H5 Uracil region, ^{1}H resonances should be shifted by 0.15 ppm upfield to obtain the actual chemical shift or for exact calculation, the upfield shift can be derived from the J_{C5H5} couplings in Hz for each residue; the values are (in Hz), U3: 176.0, U5: 176.6, U9: 176.4, U10: 177.6, U13: 177.5, U15: 177.6, U16: 178.3, U18: 175.1, U21: 175.5 and U23: 175.9. (**c**) C8H8 region, ^{1}H resonances should be shifted by ca. 0.18 ppm upfield to obtain the actual chemical shift or for exact calculation, the upfield shift can be derived from the J_{C5H5} couplings in Hz for each residue; the values are (in Hz), G1: 216.9, G2: 217.5, A6: 215.6, A8: 215.7, G11: 214.1, G14: 215.4, G17: 216.2, G20: 217.0, G22: 215.7, G24: 215.8, G26: 214.7 and A27: 216.6

determine couplings (Fig. 2). The C1′ and H1′ chemical shifts were used to extend further into the ribose rings by recording 2D and 3D experiments based on HCCH TOCSY complemented with a HCCH COSY to distinguish C2′/C3′ in order to determine C2′, C3′, C4′, H2′, H3′, and H4′ chemical shifts (Ampt, Tessari and Wijmenga, personal communication).

Aromatic proton resonances were determined for all residues, as well as C2, C5, C6 (not complete due to overlap) and C8 for the appropriate residues. No sugar resonances could be determined for G1, A6, U9, U10, G11, G17, U25 and G26 due to overlap and/or line broadening. However, the assignments of H2′, H3′, H4′, C2′, C3′ and C4′ of most of the ribose units make for a near complete assignment of the apical stem loop of epsilon of Duck HBV.

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